TITLE OF THE INVENTION

PROCESS FOR REDUCING AN ALPHA-KETO ESTER

BACKGROUND OF THE INVENTION

The invention is a process for providing high yields of an (R)-hydroxy ester by reducing an alpha-keto ester using a ketoreductase enzyme.

Simon et al., Pure & Appl. Chem., Vol. 64, No. 8, pp. 1181-186, 1992, describes properties and mechanistic aspects of redox enzymes from anaerobes suitable for bioconversions. Kataoka et al., Biochimica et Biophysica Acta 1122 (1992) 57-62, describes an NADPH-dependent aldehyde reductase (EC 1.1.1.2) isolated from red yeast Sporobolomyces salmonicolor AKU 4429, which catalyzes the reductions of D-glucuronate, D-glucose, D-xylose and D-galactose at high concentrations. Kataoka et al., Arch. Microbial. (1992) 157: 279-283, describes distribution and immunological characterization of microbial aldehyde reductases in red yeast Sporobolomyces salmonicolor AKU 4429. Kita et al., Applied and Environmental Microbiology, July 1996, p. 2303-2310, describe cloning of aldehyde reductase gene from red yeast Sporobolomyces salmonicolor AKU 4429, and characterization of the gene and its product.

Yasohara et al., Appl. Microbiol. Biotechnol. (1999) 51: 847-851, describe synthesis of optically active ethyl 4-chloro-3-hydroxybutanoate by microbial reduction using Candida magnoliae. Shimizu et al., Journal of Molecular Catalysis B: Enzymatic 5 (1998) 321-325, describes chiral alcohol synthesis with Sporobolomyces salmonicolor and Candida magnoliae yeast carbonyl reductases. Shimizu et al. Applied and Environmental Microbiology, Aug. 1990 p. 2374-2377, describes stereoselective reduction of ethyl 4-chloro-3-oxobutanoate by a microbial aldehyde reductase in an organic solvent-water diphasic system. Aldehyde reductase isolated from Sporobolomyces salmonicolor AKU 4429 and glucose dehydrogenase were used. Krix et al. Journal of Biotechnology 53 (1997) 29-39 describe enzymatic reduction of alpha-keto acids leading to L-amino acids, D- or L-hydroxy acids. The

investigation used leucine dehydrogenase and phenylalanine dehydrogenase isolated from different organisms.

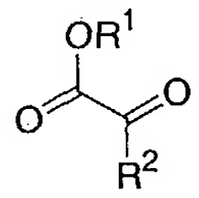
Schummer et al., Tetrahedron Vol. 47, No. 43, pp. 9019-9034, 1991, describes polyfunctional (R)-2-hydroxycarboxylic acids by reduction of 2-oxo acids with hydrogen gas or formate and resting cells of Proteus vulgaris.

SUMMARY OF THE INVENTION

The invention is a process for preparing (R)-hydroxy esters from alpha-keto esters, using an enzymatic reduction step for reducing the alpha-keto ester to the corresponding (R)-hydroxy ester. The (R)-hydroxy ester can be hydrolyzed to form the corresponding (R)-hydroxy acid, which is ultimately useful for making pharmaceutical compounds such as thrombin inhibitors.

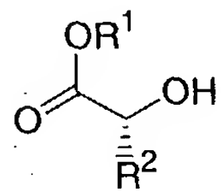
DETAILED DESCRIPTION OF THE INVENTION

In the process, the alpha-keto ester I



I

is reduced to (R)-hydroxy ester (II)



II (R)

wherein

R^1 is C_{1-4} alkyl; and

R^2 is selected from the group consisting of

C_{1-8} alkyl,

C_{1-8} alkyl, substituted with C_{3-7} cycloalkyl,

aryl, and

a 5- to 7-membered saturated or unsaturated heterocyclic ring.

The reduction is induced with a ketoreductase enzyme having a molecular weight between 36000 and 38000, and having an N-terminal amino acid sequence selected from the group of sequences consisting of

Ala-Ile-Pro-Asp-Asn-Ala-Val-Leu-Glu-Gly-Ser-Leu-Val-Lys-Val-Thr-Gly-Ala-Asn-Gly (SEQ. ID NO. 1),

Met-Ala-Ile-Pro-Asp-Asn-Ala-Val-Leu-Glu-Gly-Ser-Leu-Val-Lys-Val-Thr-Gly-Ala-Asn-Gly (SEQ. ID NO. 4),

and

Met-Ala-Lys-Ile-Asp-Asn-Ala-Val-Leu-Pro-Glu-Gly-Ser-Leu-Val-Leu-Val-Thr-Gly-Ala-Asn-Gly (SEQ ID NO. 2).

A ketoreductase enzyme having a molecular weight between 36000 and 38000, and having the N-terminal amino acid sequence Met-Ala-Ile-Pro-Asp-Asn-Ala-Val-Leu-Glu-Gly-Ser-Leu-Val-Lys-Val-Thr-Gly-Ala-Asn-Gly, is commercially available as Ketoreductase 1001 (KRED-1001) from BioCatalytics, Inc. (Pasadena, CA).

A ketoreductase enzyme having a molecular weight between 36000 and 38000, and having the N-terminal amino acid sequence Met-Ala-Lys-Ile-Asp-Asn-Ala-Val-Leu-Pro-Glu-Gly-Ser-Leu-Val-Leu-Val-Thr-Gly-Ala-Asn-Gly, has the complete sequence

Met-Ala-Lys-Ile-Asp-Asn-Ala-Val-Leu-Pro-Glu-Gly-Ser-Leu-Val-Leu-Val-Thr-Gly-Ala-Asn-Gly-Phe-Val-Ala-Ser-His-Val-Val-Glu-Gln-Leu-Leu-Glu-His-Gly-Tyr-Lys-Val-Arg-Gly-Thr-Ala-Arg-Ser-Ala-Ser-Lys-Leu-Ala-Asn-Leu-Gln-Lys-Arg-Trp-Asp-Ala-Lys-Tyr-Pro-Gly-Arg-Phe-Glu-Thr-Ala-Val-Val-Glu-Asp-Met-Leu-Lys-Gln-Gly-Ala-Tyr-Asp-Glu-Val-Ile-Lys-Gly-Ala-Ala-Gly-Val-Ala-His-Ile-Ala-Ser-Val-Val-Ser-Phe-Ser-Asn-Lys-Tyr-Asp-Glu-Val-Val-Thr-Pro-Ala-Ile-Gly-Gly-Thr-

Leu-Asn-Ala-Leu-Arg-Ala-Ala-Ala-Ala-Thr-Pro-Ser-Val-Lys-Arg-Phe-Val-Leu-Thr-Ser-Ser-Thr-Val-Ser-Ala-Leu-Ile-Pro-Lys-Pro-Asn-Val-Glu-Gly-Ile-Tyr-Leu-Asp-Glu-Lys-Ser-Trp-Asn-Leu-Glu-Ser-Ile-Asp-Lys-Ala-Lys-Thr-Leu-Pro-Glu-Ser-Asp-Pro-Gln-Lys-Ser-Leu-Trp-Val-Tyr-Ala-Ala-Ser-Lys-Thr-Glu-Ala-Glu-Leu-Ala-Ala-Trp-Lys-Phe-Met-Asp-Glu-Asn-Lys-Pro-His-Phe-Thr-Leu-Asn-Ala-Val-Leu-Pro-Asn-Tyr-Thr-Ile-Gly-Thr-Ile-Phe-Asp-Pro-Glu-Thr-Gln-Ser-Gly-Ser-Thr-Ser-Gly-Trp-Met-Met-Ser-Leu-Phe-Asn-Gly-Glu-Val-Ser-Pro-Ala-Leu-Ala-Leu-Met-Pro-Pro-Gln-Tyr-Tyr-Val-Ser-Ala-Val-Asp-Ile-Gly-Leu-Leu-His-Leu-Gly-Cys-Leu-Val-Leu-Pro-Gln-Ile-Glu-Arg-Arg-Arg-Val-Tyr-Gly-Thr-Ala-Gly-Thr-Phe-Asp-Trp-Asn-Thr-Val-Leu-Ala-Thr-Phe-Arg-Lys-Leu-Tyr-Pro-Ser-Lys-Thr-Phe-Pro-Ala-Asp-Phe-Pro-Asp-Gln-Ser-Gln-Asp-Leu-Ser-Lys-Phe-Asp-Thr-Ala-Pro-Ser-Leu-Glu-Ile-Leu-Lys-Ser-Leu-Gly-Arg-Pro-Gly-Trp-Arg-Ser-Ile-Glu-Glu-Ser-Ile-Lys-Asp-Leu-Val-Gly-Ser-Glu-Thr-Ala (SEQ ID NO. 3),

is known as Sporobolomyces salmonicolor aldehyde reductase II, and is described in Kita et al., Applied and Environmental Microbiology, Dec. 1999, p. 5207-5211.

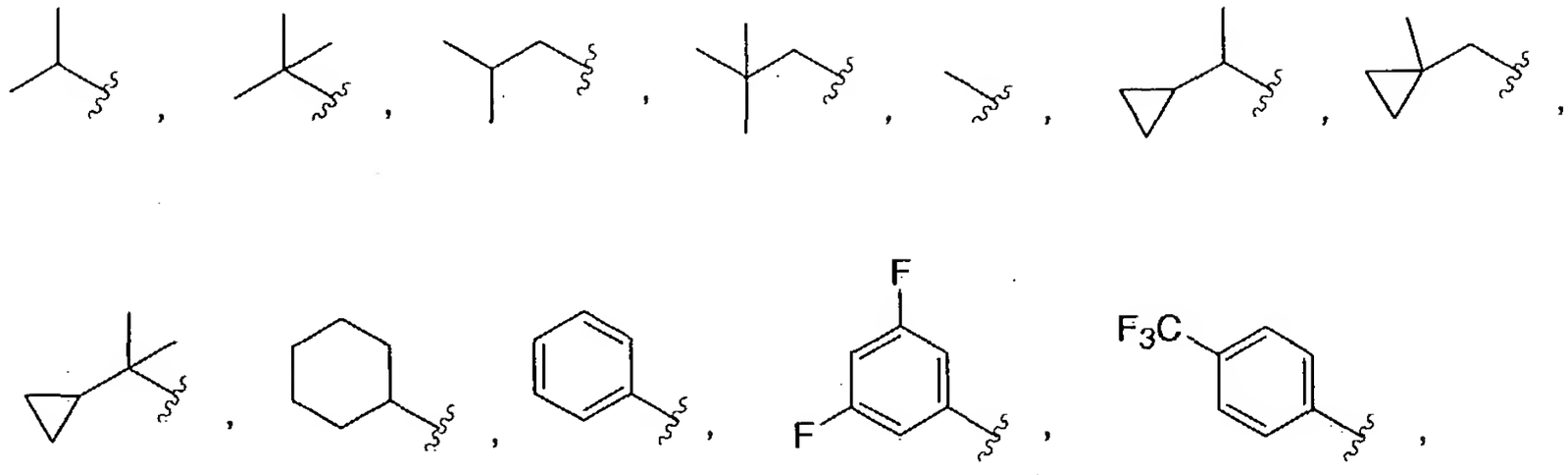
In the reduction process, NADP, an NADP/NADPH cofactor recycling system having a hydride source, such as glucose, and a catalyst, such as glucose dehydrogenase, and an appropriate buffer suitable for maintaining a pH environment of between about 5 and about 10 are used.

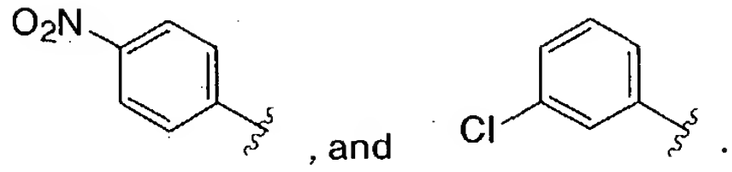
In a preferred embodiment of the process, the reduction is conducted at a temperature of between about 25 and about 40°C.

In a more preferred embodiment of the process, the amount of ketoreductase is between about 0.1 and about 10 g/L, and the amount of NADP is between about 0.1 and about 10 g/L.

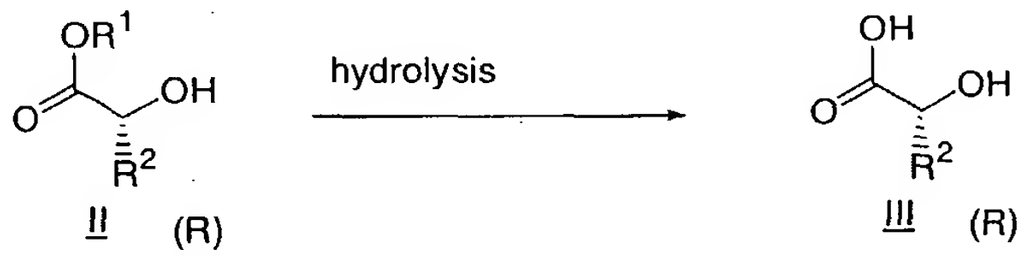
In a more preferred embodiment of the process, the hydride source is glucose and the catalyst is glucose dehydrogenase.

Specific examples of R^2 suitable for the process include





In the process, the alpha-keto ester is added to the mixture of enzyme, NADP, cofactor recycling system hydride source, and cofactor recycling system catalyst, to initiate the reaction. Examples of alpha-keto ester starting materials include ethyl 3-methyl-2-oxobutyrate (Fluka), ethyl benzoylformate (Aldrich), ethyl 3,5-difluorobenzoylformate (Aldrich), ethyl 2-(4-trifluoromethyl)-benzoylformate (Maybridge) and ethyl 4-nitrobenzoylformate (Lancaster). The mixture is maintained at a temperature between about 25 and about 40°C and agitated appropriately with attention toward providing adequate mixing of buffer and ester oil layer without inducing excessive aeration. Upon completion of the reduction reaction, e.g. about 5 hours, the (R)-hydroxy ester (II) may then be hydrolyzed, e.g. using sodium hydroxide, to form (R)-hydroxy acid (III), according to the procedure



The acid may then be extracted, using a suitable organic solvent, purified, using an appropriate purification procedure, and subsequently used in a process for making a therapeutically active ingredient such as a thrombin inhibitor.

"Cofactor recycling systems" suitable for the present invention regenerate NADPH and include systems in which the hydride source is glucose, glucose-6-phosphate, ethanol, hydrogen gas, or formate, the corresponding catalyst is glucose dehydrogenase, glucose-6-phosphate dehydrogenase, NADP dependent alcohol dehydrogenase, NADP reducing hydrogenase (methyl viologen mediated) or a variant of formate dehydrogenase which accepts NADP.

Alternatively, using a suitable alcohol substrate, the ketoreductase enzyme used in the present process for ketone reduction can be used to convert the alcohol substrate backwards to the ketone, simultaneously generating NADPH. The suitable alcohol substrates include those corresponding to ketones ethyl 3-hydroxybutanoate, ethyl 3-hydroxy-4-chlorobutanoate, and ethyl 3-hydroxy-4-phenylbutanoate, as well as 4-nitrobenzyl alcohol (to form 4-nitrobenzyl aldehyde) and pyridine-3-methanol (to form pyridine-3-aldehyde). The formed ketone or aldehyde is selectively removed, e.g., by evaporation, extraction, or reacting away, for complete conversion to the desired alcohol.

The amount of hydride required in the process depends on whether the cofactor recycling system includes reversible or irreversible steps. For example, if the cofactor recycling system includes an irreversible step (glucose as the hydride source ireversibly converts to gluconate and recycled cofactor) the hydride source is required only in slight molar excess as compared to the keto ester. If the cofactor recycling system does not have an irreversible step, the hydride source may need to be in significant molar excess, or an alternative method for recovering in situ product would be required to bring the reaction to completion.

Alternatively, in the absence of a cofactor recycling system, high concentrations of NADPH may be used directly.

The "appropriate buffer" may be any suitable buffer with a pKa between 6-9, buffering between 5 and 10. Such buffers include, but are not limited to, inorganic carbonate, phosphate, sulfite and hypochlorite bases, and organic buffers, e.g, 2-[N-Morpholino]ethanesulfonic acid, bis[2-Hydroxyethyl]iminotris[hydroxymethyl]methane, piperazine-N,N'-bis[2-ethanesulfnic acid, 3-[N-Morpholino]propanesulfonic acid, tris[Hydroxymethyl]aminoethane,

triethanolamine, N-tris[Hydroxymethyl]methyl-3-aminopropanesulfonic acid, and other common organic buffers listed on page 1873 of the Sigma catalog (e.g., HEPES, MOPS, etc.).

The term "alkyl" is intended to include both branched and straight-chain saturated aliphatic hydrocarbon groups having the specified number of carbon atoms. "Substituted" alkyl groups refer to groups having one or more defined substituents. "C_{1-4} alkyl" refers to alkyl groups having 1, 2, 3, or 4 carbon atoms, e.g. methyl, ethyl, propyl, isopropyl, etc. Likewise, "C_{1-8} alkyl" refers to alkyl groups having 1, 2, 3, 4, 5, 6, 7 or 8 carbon atoms.

The terms "cycloalkyl" and "cycloC_{3-7}alkyl" mean nonaromatic cyclic hydrocarbon groups having the specified number of carbon atoms and are intended to include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl and cycloheptyl, and the like.

The term "aryl" as used herein except where noted, represents a stable 6- to 10-membered mono- or bicyclic ring system such as phenyl, or naphthyl, wherein at least one ring is aromatic. Unless otherwise specified, the aryl ring can be unsubstituted or substituted with one or more of -CF_3, -CN, C_{1-4} alkyl, hydroxy, C_{1-4} alkoxy, halogen (e.g. F, Cl, Br, or I), -NO_2, -NR^aR^b, -SO_2R^a, $SO_2NR^aR^b$, -$CONR^aR^b$, or COR^a, wherein R^a and R^b are independently selected hydrogen and C 1-4 alkyl.

The term "halogen" includes F, Cl, Br, and I.

The terms "heterocycle", "heterocyclic", and " heterocyclyl" as used herein except where noted, represent a stable 5- to 7-membered monocyclic- or stable 8- to 11-membered fused bicyclic or stable 11- to 15-membered tricyclic ring system, any ring of which may be saturated, such as piperidinyl, partially saturated, or unsaturated, such as pyridinyl, and which consists of carbon atoms and from one to four heteroatoms selected from the group consisting of N, O and S, and wherein the nitrogen and sulfur heteroatoms may optionally be oxidized, and the nitrogen heteroatom may optionally be quaternized, and including any bicyclic group in which any of the above-defined heterocyclic rings is fused to a benzene ring. Bicyclic unsaturated ring systems include bicyclic ring systems which may be partially

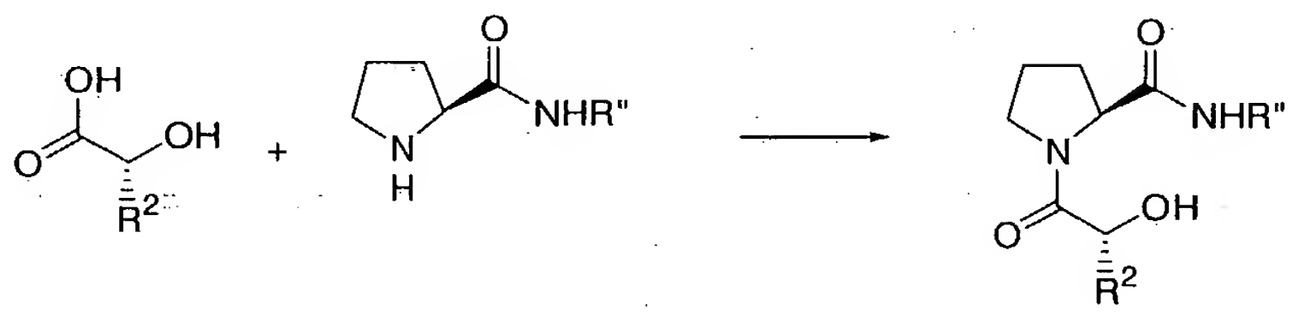
unsaturated or fully unsaturated. Partially unsaturated bicyclic ring systems include, for example, cyclopentenopyridinyl, benzodioxan, methylenedioxyphenyl groups. Especially useful are rings containing one oxygen or sulfur, one to four nitrogen atoms, or one oxygen or sulfur combined with one or two nitrogen atoms. The heterocyclic ring may be attached at any heteroatom or carbon atom which results in the creation of a stable structure. Examples of such heterocyclic groups include piperidinyl, piperazinyl, 2-oxopiperazinyl, 2-oxopiperidinyl, 2-oxopyrrolodinyl, 2-oxoazepinyl, azepinyl, pyrrolyl, 4-piperidonyl, pyrrolidinyl, pyrazolyl, pyrazolidinyl, imidazolyl, imidazolinyl, imidazolidinyl, pyridyl, pyrazinyl, pyrimidinyl, pyridazinyl, oxazolyl, oxazolidinyl, isoxazolyl, isoxazolidinyl, morpholinyl, thiazolyl, thiazolidinyl, isothiazolyl, quinuclidinyl, isothiazolidinyl, indolyl, quinolinyl, isoquinolinyl, benzimidazolyl, thiadiazoyl, benzopyranyl, benzothiazolyl, benzoxazolyl, furyl, tetrahydrofuryl, tetrahydropyranyl, tetrazole, thienyl, benzothienyl, thiamorpholinyl, thiamorpholinyl sulfoxide, thiamorpholinyl sulfone, and oxadiazolyl. Morpholino is the same as morpholinyl. Unless otherwise specified, the heterocyclic ring may be unsubstituted or substituted with C_{1-4} alkyl, halogen (e.g. F, Cl, Br and I) or NH_2.

The term "substituted," as used herein, means that any one or more hydrogens on an atom in a designated moiety is replaced with a selection from the indicated substituent group, provided that the atom's normal valency is not exceeded, and that the substitution results in a stable compound. When a substituent is an keto (i.e., =O), then 2 hydrogens on the atom are replaced.

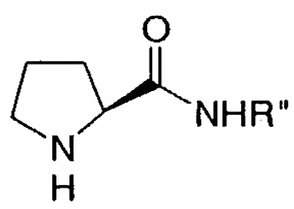
When a bond to a substituent is shown to cross a bond connecting two atoms in a ring, then such substituent may be bonded to any atom on the ring. When a substituent is listed without indicating the atom via which such substituent is bonded to the rest of the compound of a given formula, then such substituent may be bonded via any atom in such substituent. Combinations of substituents and/or variables are permissible only if such combinations result in stable compounds.

(R)-hydroxy esters resulting from reduction of alpha-keto esters may be used to prepare (R)-hydroxy acid intermediates useful in the preparation of

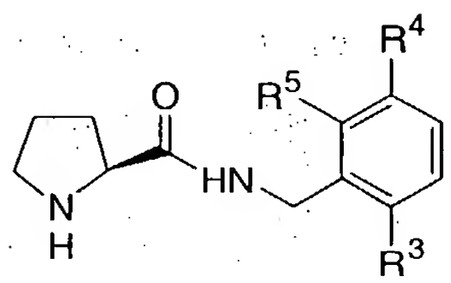
compounds, for example, by linking the carboxyl function of the (R)-hydroxy acid with a suitable nitrogen-containing compound, e.g.,



Examples of compounds that can be prepared using the (R)-hydroxy acids described above include those having thrombin inhibiting activity as determined by assays described in Thrombosis Research, Issue No. 70, page 173 (1993) by S.D. Lewis et al., e.g., compounds described in United States Patents 5,510,369, 5,629,324, 5,672,582, 5,714,485 and 5,798,377.



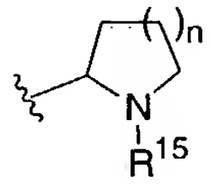
includes, but is not limited to, groups such as



wherein R^3 is selected from the group consisting of

1) $-C(R^{11})(R^{12})C(R^{13})(R^{14})N(R^{15})(R^{16})$,

2) $-C(R^{13})(R^{14})N(R^{15})(R^{16})$, and

3)  , where n is 0, 1 or 2;

wherein

R^{11} and R^{12} are independently selected from the group consisting of

a) hydrogen,

b) F,

c) C_{1-4} alkyl,

d) CF_3,

e) CHF_2,

f) C_{3-7} cycloalkyl,

or R^{11} and R^{12} together form a 3-7 membered carbocyclic ring,

R^{13} and R^{14} are independently selected from the group consisting of

a) hydrogen,

b) C_{1-4} alkyl

c) $-CF_3$,

d) $-CHF_2$,

e) $-CH_2OH$,

f) C_{3-6} cycloalkyl,

or R^{13} and R^{14} together form a 3-7 membered carbocyclic ring,

R^{15} and R^{16} are independently selected from the group consisting of

a) hydrogen,

b) C_{1-6} alkyl, unsubstituted or substituted with –OH, C_{3-7} cycloalkyl, or $C(O)OR^{19}$, wherein R^{19} is selected from the group consisting of hydrogen and C_{1-6} alkyl,

c) C_{3-7} cycloalkyl, and

d) $–C(O)R^{20}$, wherein R^{20} is selected from the group consisting of C_{1-6} alkyl, $–OR^{21}$ and $–NHR^{21}$, and wherein R^{21} is hydrogen, C_{1-6} alkyl or benzyl,

or R^{15} and R^{16} are joined to form a 4-7 membered heterocyclic ring which is unsubstituted or substituted with hydroxyl or halogen;

R^4 is halogen; and

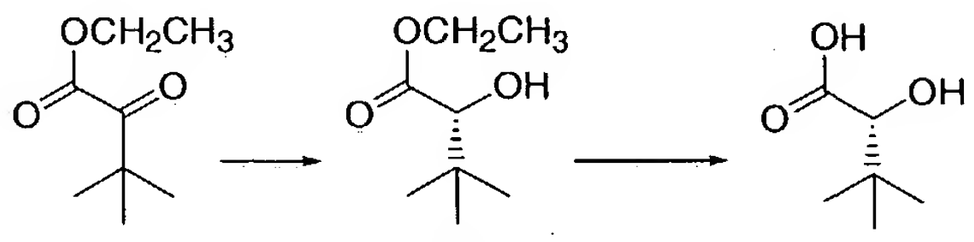
R^5 is hydrogen or halogen.

Thrombin inhibitors are useful in anticoagulant therapy for the treatment and prevention of a variety of thrombotic conditions, particularly coronary artery and cerebrovascular disease. Those experienced in this field are readily aware of the circumstances requiring anticoagulant therapy. Thrombin inhibitors are useful for treating or preventing venous thromboembolism (e.g. obstruction or occlusion of a vein by a detached thrombus; obstruction or occlusion of a lung artery by a detached thrombus), cardiogenic thromboembolism (e.g. obstruction or occlusion of the heart by a detached thrombus), arterial thrombosis (e.g. formation of a thrombus within an

artery that may cause infarction of tissue supplied by the artery), atherosclerosis (e.g. arteriosclerosis characterized by irregularly distributed lipid deposits) in mammals, and for lowering the propensity of devices that come into contact with blood to clot blood. The thrombin inhibitors can be administered in such oral forms as tablets, capsules (each of which includes sustained release or timed release formulations), pills, powders, granules, elixers, tinctures, suspensions, syrups, and emulsions. Likewise, they may be administered in intravenous (bolus or infusion), intraperitoneal, subcutaneous, or intramuscular form, all using forms well known to those of ordinary skill in the pharmaceutical arts. The compounds can also be administered in intranasal form via topical use of suitable intranasal vehicles, or via transdermal routes, using those forms of transdermal skin patches well known to those of ordinary skill in that art. The dosage regimen utilizing the thrombin inhibitors is selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the patient; the severity of the condition to be treated; the route of administration; the renal and hepatic function of the patient; and the particular compound or salt thereof employed. An ordinarily skilled physician or veterinarian can readily determine and prescribe the effective amount of the drug required to prevent, counter, or arrest the progress of the condition.

The following examples are illustrative of the invention but should not be interpreted as limiting the scope of the invention as defined above.

Example 1



1-1 (R) 1-2 (R) 1-3

6.4 L of a 200mM K_2HPO_4 buffer (544.64 grams in 10 L of Millipore water), 448 grams of glucose, 3.2 grams of glucose dehydrogenase, 0.736 grams of NADP, 0.64 grams of KRED 1001 (Biocatalytics, Inc., Pasadena, CA) ketoreductase enzyme, were added to a mixing vessel in order to form a mixture. 295.2 grams of

ethyl trimethylpyruvate ($(CH_3)_3C(O)C(O)OCH_2CH_3$) 1-1 was added to the mixture to form a final volume of 6.4 liters. The reaction was allowed to proceed at 25°C and 222-223 rpm. The pH was controlled at 7.0 using NaOH (5-20% v/v). The reaction proceeded for 5 hours to form ester (R) 1-2. After bioconversion was completed, concentrated NaOH (50% v/v) was poured into the reaction solution in a 1:10 ratio. The final volume of the reaction (including the addition of 5% NaOH during pH control) was approximately 7.2 L.

Example 2

Example 1 is repeated, except that the glucose/glucose dehydrogenase cofactor recycling system is replaced with a glucose-6-phosphate/glucose-6-phosphate dehydrogenase cofactor recycling system, wherein the amount of glucose-6-phosphate hydride source is used in molar amounts in slight excess to molar amounts of the keto ester.

Example 3

Example 1 is repeated, except that the glucose/glucose dehydrogenase cofactor recycling system is replaced with an ethanol/NADP dependent alcohol dehydrogenase cofactor recycling system, wherein the amount of ethanol hydride source is used in molar amounts in great excess to molar amounts of the keto ester.

Example 4

Example 1 is repeated, except that the reaction proceeds for 10 hours to form ester (R) 1-2.

Example 5

Example 1 is repeated, except that the reaction proceeds for 16 hours to form ester (R) 1-2.